Investigating the effect of Copper (CuI) depletion on the serotonergic neurons and serotonin signalling in the nematode based model.

Project report submitted to Sister Nivedita University in partial fulfilment of the requirement for the degree of

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IN
BIOTECHNOLOGY

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Dissertation Completion Certificate

To whom it may concern

This is to certify that Ms. Iram Khan, from the Department of Biotechnology, Sister Nivedita University, has successfully completed her dissertation at Amity University, Kolkata, India, from January 7, 2025, to April 7, 2025, under my supervision. Her research area included cellular metal homeostasis and its role in neurodegeneration.

Her project, titled "Investigating the Effect of Copper [Cu(I)] Depletion on Serotonergic Neurons and Serotonin Signalling in a Nematode-Based Model," represents a significant contribution to the field and is a record of her original research work. Throughout the course of this study, she has exhibited strong analytical skills, scientific rigor, and dedication to her work. Her work demonstrates a clear understanding of the subject.

I hereby acknowledge her successful completion of this dissertation under my supervision.

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DECLARATION BY THE STUDENT

I hereby declare that the work which is being presented in this dissertation entitled "Investigating the effect of Copper (CuI) depletion on the serotonergic neurons and serotonin signalling in the nematode based model" in partial fulfilment of the requirements for the award of the degree of Masters of Science in Biotechnology is an authentic record of my own work carried out during the period from 7th Jan to 7th April under the supervision of Dr. Ashima Bhattacharjee , Associate Professor , Amity Institute of Biotechnology ,Amity University Kolkata.

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TO WHOM IT MAY CONCERN

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ABSTRACT:

Copper is a trace element required for a number of biological processes such as neurotransmitter production, mitochondrial respiration and antioxidant production. Furthermore, disturbances in copper homeostasis have also been associated to neurodegenerative processes and to neuronal signalling. Cu(I) depletion in the Menkes Disease patients is characterized by neurodegeneration underlining the importance in relation to neurons. Analysis of the role of copper in the various neuronal types and subtypes is difficult because the CNS architecture is highly complicated in multicellular organisms. The dissertation aims to investigate the role of copper on the individual signalling systems in the simple *C. elegans* model system. The purpose of this study is to investigate the effect of copper (I) depletion on serotonergic neurons and serotonin signalling in *C. elegans*. It is a model organism with a well-characterized neuronal system and molecular pathways. Consequently, it can be utilized to study the relationship between copper metabolism and neuronal degeneration. We used TTM (tetrathiomolybdate) to deplete copper levels in *C. elegans*. Copper depletion has a differential effect on the serotonergic neurons. Serotonergic neurons (HSN) manifest degenerative phenotype in the form of broken axonal extensions.The worms show decreased egg laying possibly due to decreased serotonin release by the HSN neuron into the vulval muscles. Therefore, the conducted work indicates the main function of copper in promoting the serotonergic neuronal health and function.

KEYWORDS:

Copper homeostasis, *C.elegans*,Confocal microscopy, Serotonergic neurons,TTM, Nematode, Copper chelation,copper,HSN neurons.

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Abbreviations:

NGM- Nematode Growth Media

RDA- Recommended Dietary Allowance

DMSO- Dimethyl sulfoxide

TTM- Tetrathiomolybdate

CTR- Copper transporter

GI- Gastrointestinal Tract

ATOX- Antioxidant 1 Copper Chaperone

COX- Cytochrome C Oxidase

CCS- Copper chaperons

IMS- Mitochondrial Intermembrane Space

ADF-Amphid neuron with dual ciliated sensory endings

NSM- Neurosecretory Motor Neurons

HSN- Hermaphrodite-Specific Neurons

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1. INTRODUCTION:

Copper is a critical trace element because it plays prominent roles in a number of physiological processes required for normal development, growth and maintenance of health (Chen et al., 2022). Copper, an essential trace metal, is a component of a range of physiological processes including cellular respiration, iron metabolism, and antioxidant defence, as well as neuropeptide production (Yruela et al., 2009). For healthy people without known liver disease, the recommended dietary allowance of copper is approximately (900 micrograms) per day for adolescents and adults. Pregnant and lactating women have higher copper requirements that should be at least 1300 ug from dietary sources each day (Kumar Sowmya, Binu,2024) Copper is regulated and maintained in various organs and cells of our body using different copper chaperones and transporters. Dysregulation of copper has been seen to cause many consequences like neurodegeneration.

1.1. Sources in the body

It is found in many foods, including water, organ meat like kidney and liver, shellfish like oysters, nuts and seeds, whole grains like wheat-bran cereals, dark leafy greens, dried fruits like cocoa, fruits like avocados, guavas, pomegranate, blackberries, kiwi, mangoes, etc. Black pepper, Yeast, a dried supplement made from blue-green algae, mushrooms like white button mushrooms, and chocolate are also good sources of copper. Apart from this, using copper utensils is also a good source of copper intake in body. Body acquires copper through diet and drinking water.

1.2. Importance

It is also a co-factor for several enzymes known as cuproenzymes which catalyze important biological processes such as energy production, iron metabolism,activation of brain neuropeptides and formation of connective tissue, brain neurotransmitters (Turski et al., 2009).These Cuproenzymes are involved in a range of biological functions and therefore speak to the importance of a functional copper status of the organism in maintaining overall health and wellbeing (Turski et al., 2009). Benefits of Copper as a cofactor Copper is also a key cofactor for many critically important enzymes, which includes cytochrome c oxidase (energy production), and superoxide dismutase (detoxification of superoxide, reducing the toll of oxidative damage for the organism). Copper is also responsible for stimulating the entry of iron into the red blood cells. Copper and its co-factors are necessary for the creation of hemoglobin and this metal is

also part of certain regulatory Copper is a cofactor which can come to form part of proteins in the body such as tyrosinase, that help to maintain proper body function (a similar function is shared by iron). Dopamine beta hydroxylase is a copper dependent enzyme that helps in the synthesis of neurotransmitters by converting dopamine to norepinephrine. Copper containing enzymes help to scavenge free radicals and protect cells from damage. Copper is required for synthesis of collagen and elastin which provide structure and support to connective tissue. Copper contributes in the absorption and utilization of iron. Adequate copper levels are necessary for normal iron metabolism, red blood cell production, and function. Cu depletion can result in impaired iron metabolism. It is required for the production of, norepinephrine, epinephrine, all of which are important neurotransmitters. Copper helps in neuronal differentiation (**Chakraborty et al., Metallomics,2022).**

It plays an important role in neuropeptide maturation, brain development, and regulation of gene expression(**Rajaa El Meskini, Kelli L. , Laura B. et al., 2007)**. It is essential for proper brain function and development4. Copper is also involved in immune system functioning and defense against oxidative damage. It plays a role in protecting cells from oxidative stress and maintaining overall immune health. Copper, as well as enzymes containing it, such as superoxide dismutase, plays a role in the dismutation of superoxide, in defense against oxidative stress. Cu is also necessary for functions such as angiogenesis (the process of making new blood vessels) and pigmentation. Cu also serves as a vital part in the catalytic activity of copper and it has a unique chemical property owing to its ability to be either in Cu1 (cuprous) or Cu2+ (cupric) oxidized state (Fahrni et al.,2013). Although it is necessary, copper concentration must be tightly controlled in the body and in different type of cells. This fact is fuelled by the prevalence of multiple diseases caused by dysregulation of copper homeostasis(Ashima Bhattacharjee,Kaustav Chakraborty et al.,2017).

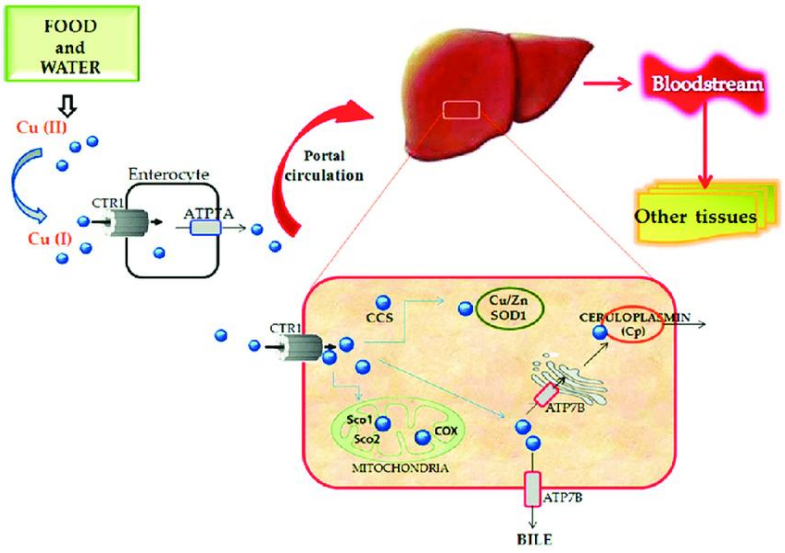


Fig.-1 Copper absorption, distribution, and metabolism. **Antonucci et al. 2017**

1.3. How copper homeostasis is maintained in the body

Homeostasis of copper in mammals is achieved

through intricate mechanisms that regulate its absorption and excretion.. Excretion of this metal ion is achieved by intracellular flux control, copper import and efflux across the intestinal basolateral membrane.

This element enters our bodies primarily through food. Copper homeostasis is regulated in the body through the liver and the gastrointestinal (GI) tract (**Ashima Bhattacharjee,Kaustav Chakraborty et al.,2017).**

Copper enters the body through the mouth and travels through the oesophagus and enters the enterocytes forming the intestinal linning. It enters the enterocytes with through plasma membrane copper transporter CTR1 and enters the cytosol. It crosses the cell membrane and binds to a number of copper chaperones (ATOX1, CCS, and COX17) that are responsible for transporting copper to various intracellular compartments. There, ATOX1 transports Cu to the trans-Golgi network membrane ATPases, ATP7A and ATP7B. These transporters mediate uptake of copper inside the lumen of the trans-Golgi network, where it can be loaded onto various cuprous proteins (secretory pathway). When intracellular copper levels are elevated, ATP7a and ATP7b are both recruited to endocytic vesicles, which drive towards the apical and basolateral surface respectively The vesicles fuse with the plasma membrane and expel excess copper from the cell. However in unpolarized cells, it has been shown that ATP7b vesicles traffic towards vesicles. The vesicles ultimately fuse with the lysosomes and excess copper is exported out of the cells through lysosomal exocytosis (**Ashima Bhattacharjee,Kaustav Chakraborty et al.,2017).**

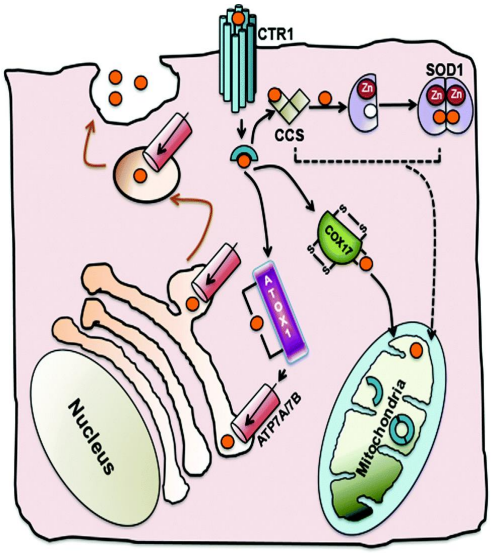


Fig.-2 Cellular copper homeostasis: an overview. After entering the cell through copper transporter CTR1, copper (orange sphere) binds with glutathione. Copper is then transferred to several intracellular chaperones like ATOX1 (purple rectangle), COX17 (green hemisphere) and CCS (shaded diamond). CCS delivers copper to SOD1 both in the cytosol and the mitochondrial intermembrane space (dotted lines and arrows). ATP7A/7B (red cylinder), residing on TGN, receive copper from ATOX1. ATP7A/7B traffic to vesicles (spheres containing ATP7A/7B) under high intracellular copper, thereby exporting excess copper out of the cell. Cellular glutathione (GSSG, oxidized– blue ring, GSH, reduced– blue half ring) has been demonstrated.

Bhattacharjee et al, Metallomics, 2017.

Diet and drinking water are the sources of copper in the body. Copper is absorbed into the intestinal enterocytes and reaches the bloodstream through ATP7A mediated export. ATP7A traffics to the basolateral plasma membrane in the intestinal enterocytes and exports copper into the bloodstream. The copper in the blood is then transported to various organs. Liver plays a major character in copper excretion. ATP7B is the only copper transporter in the hepatocytes. ATP7B traffics to the apical plasma membrane and exports excess intracellular cu into the

bile canaliculi. This copper is ultimately excreted out of the body through faecal matter.

1.4. Cellular copper homeostasis

Process by which organism regulate Cu(I) level within their individual cells is called cellular copper homeostasis. Copper is taken up into the cell by a high-affinity copper transporter CTR1, which is a trimeric protein. Copper freely interconverts between Cu(I) and Cu(II). However, in reducing intracellular condition, copper will be left mostly in the Cu(I)-form. It enters the cell and then binds to a number of chaperones which have the role of shuttling the copper to various intracellular locations. Once inside the cell Cu(I) has four possible routes. Copper chaperone for superoxide dismutase (CCS) helps in the maturation and copper loading of Cu–Zn superoxide dismutase-1 in the cytosol. CCS-induced maturation and copper loading for SOD1 occur in the IMS of mitochondria and particulate SOD1 enzymes are distributional in the two compartments. Thus CCS has two functions in copper trafficking to SOD1 between cytosol and mitochondrial IMS Copper chaperone, COX17, mediates copper delivery and cytochrome C oxidase (COX) biogenesis and is also related with the maintenance of mitochondrial copper homeostasis. Cytosolic chaperone ATOX1 binds Cu(I) and delivers it to the ATPases, present on the membrane of the TGN. These transporters deliver Cu(I) into the lumen of the trans-Golgi network using the energy derived from ATP hydrolysis. The transported Cu(I) is incorporated into several cupro-proteins (**Ashima Bhattacharjee,Kaustav Chakraborty et al.,2017).**

When the intracellular copper is high, both ATP7A and ATP7B are relocalized to endocytic vesicles, which are then transported and finally fused to the plasma membrane to export the excess copper out of the cell (Figure. 1). Copper is also translocated to theMitOchondrial intermembrane space (IMS) by CCS (complete full name) for Cu(I) entry into Superoxide Dismutase. CCS serves a double duty in targeting copper to SOD1 within the cytosol and mitochondria. Copper is also transported into the nucleus but the pathway is unknown. Cu(I) chaperone COX17, delivers Cu(I) into the lumen of the mitochondria for and maturation of cytochrome C oxidase (COX) and plays an important role in mitochondrial copper homeostasis (Ashima Bhattacharjee,Kaustav Chakraborty et al.,2017); (**Fig.2**).

1.5.Copper metabolism disorder

Perturbation of the cellular copper homeostasis will lead to failure of the cellular process.. Mutational inactivation of the ATP7A results into the failure of delivery of copper into the blood stream there by causing systemic copper deficiency and leading to Menkes Disease(Tina

Skjørringe, Per Amstrup et al. 2017.). Mutation inactivation of the hepatocellular predominant Cu(I) transporter ATP7B results in the inability of Cu(I) egressing from the hepatocyte into the biliary system. This exceeded Cu(I) gets deposited in Liver and different other organs e.g. brain and kidney which can cause the Wilson Disease.

1.5.a. Menkes Disease

Menkes disease, which is also called Menkes syndrome, is a very rare X-linked recessive genetic disorder of copper metabolism. Menkes disease is a mutation of the ATP7A gene encoding a copper-transporting ATPase that is involved in copper efflux from the cell to the blood. Failure of this transport process causes systemic deficiency of copper, particularly in brain and nervous system where the element is vital for the operation of a variety of enzymes involved in neurotransmitter synthesis, antioxidant protection and other vital processes. Menkes disease is predominantly seen in males, with symptoms usually manifesting at infancy, resulting in serious developmental delay, neurological deterioration, and shortened life span, usually not surviving beyond 3 years of age (Tümer & Møller, 2010).

a.1. Clinical representation and Pathophysiology

The clinical representation of Menkes disease is multifaceted and mirrors the systemic nature of copper deficiency. Frequent findings include kinky, brittle hair (pili torti), failure to thrive, neurologic deterioration, and intellectual impairment. Menkes disease infants are usually diagnosed with hypothermia, hypoglycemia, prolonged jaundice, and seizures, which are usually apparent to parents around 6-8 weeks of age. The disorder is also characterized by labile body temperature, prematurity, and delayed development. The neurologic sym ptoms of seizure and abnormal movement of the limbs or eyes are especially disabling and result from deficient copper-dependent enzymatic activity within the brain (**Tümer & Møller, 2010**).The mutation of the ATP7A gene inhibits the normal function of the copper-transporting ATPase, and as a result, there is a loss of supply of copper to copper-requiring enzymes like cytochrome c oxidase, dopamine β-hydroxylase, and lysyl oxidase. These enzymes are important in energy metabolism, synthesis of neurotransmitters, and the development of connective tissue, respectively. Enzyme deficiency results in the clinical complexities of Menkes disease, which are neurodegeneration, connective tissue defects, and vascular malformations (**Horn & Tümer, 2000).**

a.2. Genetic and Molecular Basis

Menkes disease is a recessive disorder linked to the X chromosome, caused by mutations in the ATP7a gene. Heterozygous males, possessing one X chromosome, are mainly affected, whereas heterozygous females possessing two X chromosomes are usually carriers and can have milder manifestations due to X-inactivation. The Atp7A gene encodes a P-type ATPase transporting the copper across the cell membranes such as intestinal epithelium to take up the copper and across the blood-brain barrier to facilitate delivery of copper to the central nervous system (Horn & Tümer, 2000).The Atp7a mutations are as varied as missense mutations to deletions of numerous base pairs, and in general, the severity of the disease will depend on the nature of the mutation and where the mutation is located. Mutations leading to total loss of ATP7A activity lead to the classical, severe Menkes disease, while milder mutations can lead to atypical instances of the disease with delayed onset and minimal symptoms (Tümer & Møller, 2010).

a.3. Diagnosis and Treatment

Menkes disease diagnosis depends on clinical presentation, biochemical screen, and genetic investigation. Lower blood levels of cu and ceruloplasmin are classic biochemical characteristics of the condition. Confirmation is made by genetic testing to detect mutation in the ATP7A gene (**Tümer & Møller, 2010**).Menkes disease has little treatment and only symptom-oriented care mainly. Nevertheless, early treatment with parenteral copper-histidine supplementation has shown promise in altering the disease course and reducing symptoms. Copper-histidine treatment is designed to circumvent impaired intestinal absorption of copper and transport copper directly into tissues and copper-dependent enzymes. Early treatment with copper-histidine, preferably within the first few weeks of life, has been linked to enhancing neurological function and survival in a subset of patients, according to studies. But the efficacy of this treatment is reduced if it is begun after the onset of significant neurological symptoms (**Tümer & Møller, 2010**).Studies on Menkes disease have shed light on the function of copper in neurodevelopment and neurodegeneration. **Zeynep Tümer and Lisbeth B. Møller (2010**) have emphasized the necessity of early diagnosis and treatment in Menkes disease and the therapeutic potential of copper-histidine therapy in reducing the severity of the disease. Furthermore, Horn and Tümer (2000) have explained the molecular mechanism of Menkes disease, with the function of ATP7A in the transfer of Cu and its influence on copper-dependent enzymes.

Menkes Disease is a lethal gene disorder that is a powerful illustration of the importance of copper for brain and general health. Aberration of ATP7a gene causes abnormalities in copper transport, resulting in systemic copper deficiency and devastating neurodevelopmental consequences. Although treatment options are restricted, copper supplementation with histidine appears promising for disease reversal.

Further investigation into the molecular pathophysiology of Menkes disease and copper metabolism may also result in improved treatment options and improved knowledge of the significancof copper in neurodegenerative illness.

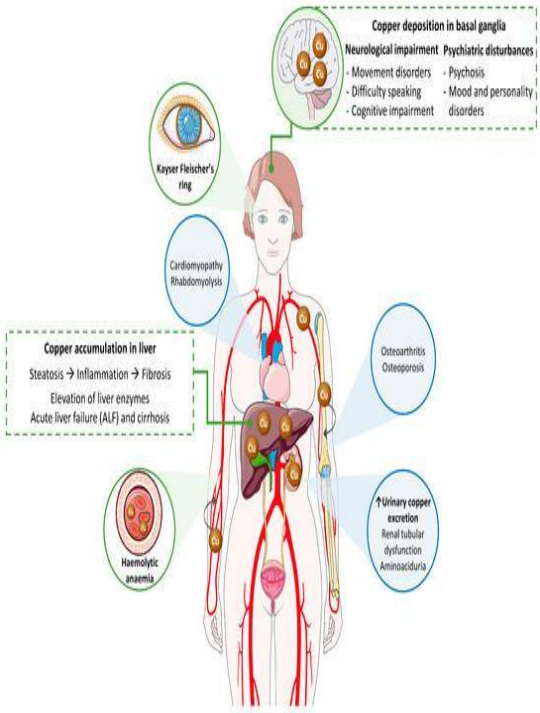


Fig- 3.Monteagudo et al.,Facing the Challenge of Diagnosing a Rare Disease,**2021.**

1.5.b. Wilson's Disease

Wilson's disease is a genetic disorder in the autosomal recessive mode due to abnormal accumulation of cu in the body. It is also known as hepatolenticular degeneration and is a rare genetic disorder of copper deposition within tissues, with the liver, brain, and kidneys being affected most commonly. The disease is caused by mutations in the ATP7B gene, coding for a copper-transporting ATPase that is responsible for the excretion of excess metal element into bile. Incorporation of remaining Cu into ceruloplasmin is also impaired by the deficiency in the ATP7B gene. of copper homeostasis, which results in its toxic deposition in organs and further tissue injury (**Sánchez-Monteagudo, Ripollés, & Berenguer, 2021**). Wilson's disease is a chronic condition that, in the absence of treatment, may lead to severe hepatic, neurological, and psychiatric morbidity. But through early diagnosis and proper treatment, normal life can be resumed by patients.The clinical presentation of Wilson's disease is extremely unpredictable and is based on the organs in which copper accumulates. The disease presents in one of three forms, i.e., hepatic, neurological, or psychiatric.

b.1. **Hepatic Symptoms:**

The liver is frequently the initial organ to become involved, especially in children. The symptoms at presentation could be hepatomegaly, jaundice, chronic hepatitis, and cirrhosis. Acute liver failure in a few patients is presented by the patient with coagulopathy, hemolysis, and encephalopathy. Liver shows signs is frequently the initial presentation of Wilson's disease and can occur before neurological or psychiatric presentation by a few years (**Sánchez-Monteagudo et al., 2021).**

b.2. Neurological Symptoms:Neurological manifestations typically become apparent during the second or third decade of life and comprise tremor, dystonia, dysarthria (impaired speech articulation), ataxia

(failure of coordination), and parkinsonian features like rigidity and bradykinesia. These manifestations occur as a consequence of copper deposition in basal ganglia and other locations in the brain and result in neurodegeneration (**Gaggelli, Kozlowski, Valensin, &Valensin, 2006**).

b.3. Psychiatric Symptoms

Psychiatric symptoms are frequent and can be depression, anxiety, mood swing, psychosis, and cognitive impairment. The symptoms can be confused with primary psychiatric disorders and result in a delay in the appropriate diagnosis of Wilsons disease (Sánchez-Monteagudo et al., 2021). Pathophysiology Wilson's disease involves the fact that the protein ATP7B cannot export copper properly. In normal circumstances, ATP7B enables the removal of excess copper into bile and delivers copper to ceruloplasmin, a copper carrier protein. In Wilson's disease, ATP7B mutations disrupt these processes, causing copper buildup in hepatocytes. With prolonged duration, copper permeates blood & becomes deposited in other organs as the brain, kidneys, & cornea, and generates oxidative stress and cell damages (Gaggelli et al., 2006).

b.4. Diagnosis

Diagnosis of Wilson's disease can be difficult because of the heterogeneity of the presentation of the disease and also because it can imitate various diseases. These below mentioned tests are used to diagnose:

Biochemical Tests:

Reduced serum ceruloplasmin: Ceruloplasmin is usually low in Wilson's disease because of the abnormal incorporation of copper.Elevated 24 hour urinary excretion of copper. It is characteristic of Wilson's disease and indicates the attempt of the body to get rid of excess copper.Elevated hepatic copper content: Histological examination of liver biopsy demonstrating content of more than 250 µg/g dry weight is diagnostic (**Sánchez-Monteagudo et al., 2021**).

Genetic Tests:

Identification of mutations of the ATP7B gene is diagnostic and can be very helpful in screening relatives from affected families because Wilson's disease has autosomal recessive inheritance.

Supportive Tests :

Kayser-Fleischer rings: Deposits of copper within the cornea, visible on slit-lamp examination, are found in the larger part of patients with neurological symptoms.

Brain imaging: MRI will reveal typical changes in basal ganglia, e.g., hyperintensities on T2-weighted images (**Gaggelli et al., 2006**).

b.5. Treatment : Treatment aims at decreasing storage of copper in the body and inhibiting future deposition. Treatment comprises:

Chelating Agents:

D-penicillamine: It induces mobilization of this trace metal ion in the urine but may produce severe side effects such as bone marrow suppression and nephrotoxicity.Trientine: Less-toxic chelator,trientine is generally administered to those unable to tolerate D-penicillamine **(Sánchez-Monteagudo et al., 2021).**Zinc Supplements prevents its absorption in the intestine by stimulating metallothionein, a copper-sequestering protein in enterocytes. Zinc is typically used as maintenance therapy or in combination with chelating drugs (**Gaggelli et al., 2006).**

Dietary Changes : Patients are requested to exclude copper-containing foods like shellfish, nuts, and chocolate, and to employ distilled or demineralized water if the water supply is rich in copper.Early diagnosis and long-term compliance with therapy are essential to avoid disease progression and complications. Non-compliance with therapy may result in irreversible liver damage, deterioration of the neurological state, and death (**Gaggelli et al., 2006**).Studies in Wilson's disease have yielded useful insights into copper metabolism and its involvement in neurodegenerative diseases. There is a great emphasis being laid on the significance of copper homeostasis in the brain and its disturbance in Wilson's disease compared to other neurodegenerative diseases like Alzheimer's and Parkinson's diseases **(Gaggelli et al. (2006)**. The research indicates the involvement of oxidative stress in neurodegeneration by copper and the therapeutic potential by antioxidants.Researchers are putting great emphasis on the challenge of diagnosing Wilson's disease, especially when it is in its early stage and symptoms can be nonspecific. (**Sánchez-Monteagudo et al. 2021**).They reiterate the importance of better awareness among clinicians and the use of standardized diagnostic protocols to enhance early diagnosis and patient outcome.Wilson's disease is a genetically multifactorial disorder that highlights the importance of copper homeostasis to enable organs to function. Genetic mutations in the ATP7B gene result in copper accumulation and cause hepatic, neurological, and psychiatric manifestations. Early detection and prolonged treatment with chelating drugs, zinc supplementation, and dietary adjustment are essential to avoid progression of the disease and complications. Research into the molecular basis of Wilson's disease and copper metabolism continues to show promise for the development of more efficient therapies and knowledge of its association with other neurodegenerative diseases.

1.5.c. Alzheimer’s Disease

Alzheimer's disease (AD) is a progressive neurodegenerative diseasecharacterizedby cognitive dysfunction, memory loss, and behavioral changes. Azd is common form of dementia and is widespread

across millions of people worldwide. The pathological hallmarks in Ad are deposition of amyloid-beta plaques, neurofibrillary tangles composed of hyperphosphorylated tau protein, and severe neuronal loss. Recent studies, however, emphasize the pivotal role of neuroinflammation and immune dysregulation in the pathogenesis of the disease. A new study **(Schneeberger et al. ,Nature Aging,2025)**illuminates the role of Interleukin-12 (IL-12) signaling in the pathology of Alzheimer's disease. The authors show that IL-12 perturbs neuronal and oligodendrocyte homeostasis, leading to neurodegeneration and myelin dysfunction. This result emphasizes the relevance of immune-mediated processes in AD and implies that targeting IL-12 signaling may provide a new therapeutic strategy to prevent disease progression. Recognition of the interaction among neuroinflammation, health of neurons, and oligodendrocyte function will be essential to creating effective therapeutic interventions for this debilitating disease.

1.5.d. Parkinson's Disease

The progressive neurodegenerative disease known as Parkinson's disease (PD) causes non-motor symptoms including cognitive decline and mood disorders as well as motor symptoms such as tremor, rigidity, and bradykinesia due to the loss of neurons that produce dopamine in the substantia nigra. Studies show how important copper ions are to Pd pathogenesis. Maintaining appropriate cellular activity depends on copper homeostasis—that is, control of copper ion levels within the body. All of which are linked to the neurodegeneration seen in PSd, disruptions in copper homeostasis can cause oxidative stress, protein misfolding, and mitochondrial dysfunction. Therefore, developing therapeutic approaches to slow down the development of Parkinson disease depends on an awareness of the processes behind copper homeostance. This work emphasizes the need of looking at molecular targets, like copper control, for PD disease-modifying treatments**.(Bisaglia, M., &Bubacco, L. ,2020).**

1.5.e. Occipital Horn Syndrome

Occipital horn syndrome (OHS) also called X-linked cutis laxa and Ehlers-Danlos syndrome, type IX, is an ultra-rare recessive genetic disease of connective tissue, and is among the disorders of cu metabolism due to mutation of the ATP7A gene. OHS is also an X-linked recessive disorder like Menkes disease, although milder to manifest. OHS has the usual characteristic skeletal abnormalities, such as occipital horns (osseous outgrowths at the posterior aspect of the cranium), defective connective tissue, and with more or less mild neurological manifestations. OHS caused by mutations in gene ATP7A which encodes a copper-transporting ATPase required for copper homeostasis. But in contrast to classical menkes disease, OHS is caused by deep intronic mutations inducing pseudo-exons that lead to partially functional ATP7A protein (**Yasmeen et al., 2013**).

Clinical presentation of OHs differs from classical Menkes disease and expresses the lesser effect of the ATP7A mutations. Important features are:

e.1. Skeletal Abnormalities:

Occipital horns: Bony spurs along the posterior skull base, pathognomonic of OHS.

Loose joints: Hyperhydricity and joint laxity are likewise observed in OHS patients, as in other disorders of connective tissues.

Connective Tissue Defects:Cutis laxa, Hernias .

Scoliosis and Vascular disorders: The walls of the blood vessels are compromised and lead to vascular complications (**Yasmeen et al., 2013).**

e.2. Neurological Symptoms:

Mild intellectual disability: Mental impairment will be less severe than in Menkes disease.Muscle weakness: Hypotonia and muscle weakness are noted but less disabling than in classical Menkes disease (**Yasmeen et al., 2013**).The etiology of OHS is attributed to partial dysfunction of the ATP7A protein. In OHS, deep intronic mutations within the ATP7A gene activate pseudo-exons, splicing errors leading to the incorporation of non-coding DNA fragments in the mRNA. This produces a partially functional ATP7A protein with residual copper-transporting activity, thus accounting for the less severe phenotype than that seen in Menkes disease (**Yasmeen et al., 2013**).

e.3. **Diagnosis :** Diagnosis of OH made on the basis of clinical presentation, biochemical tests, & genetic studies: Cutis laxa, occipital horns, and joint hypermobility are highly suggestive of OHS.BiochemicalInvestigations:Low serum and ceruloplasmin: These are typical findings, although they are milder than in Menkes disease.Urinary excretion of copper: Indicates disturbed transport of copper (**Yasmeen et al., (2013**).

e.4. Treatment : Copper-histidine therapy , Hernia repair: Herniorraphy can be required.Orthopedic management: Joint deformities and scoliosis may require surgical or non-surgical management .

2. Origin of proposal

1. Cu(I) is critical during embryogenesis[4]. Depleted copper availability during embryogenesis leads to a lethal defect and abnormality in the notochord formation in zebrafish suggesting a pivotal significance of this ion during the development (**Bryce A Mendelsohn , Chunyue Yin;2006**). In case of Mottle Brindled mice (MNK model) ,extremely low amount of cu in brain leads to abnormal axon and dendrite formation leading to distorted synaptogenesis and major neuropsychiatric manifestations before lethality and very

early death(**Tina Skjørringe,2017**). Published data from this laboratory reports that copper chelation through TTM (Tetrathiomolybdate) treatment effects neurite generation of the PC-12 derived neurons (**Chakraborty et al., Metallomics,2022**).

All these studies demonstrate the crucial importance of Cu(I) towards neurite development, neuronal differentiation and synaptogenesis in the CNS. Further unpublished studies from the laboratory demonstrates that copper chelation causes degeneration of the dopaminergic neurons in *C.elegans* model. Therefore we aimed to invetsigate the contribution of depletion on the serotonergic neurons in this inorder to understand its role in the serotonergic neurons and serotonergic signalling.

2.5. *C.elegans* as a model for studying neuronal differentiation

C. elegans is a microscopic transparent roundworm. Reasons which make it a good model organism are as follows:

1. **Genetic simplicity-** simple as compared to higher organism like mammal.
2. **All 302 neurons of *C. elegans*** - Characterized & genetically modified with fluorescent proteins for ease of study.
3. **Short reproductive cycle-** the life cycle of microscopic worm is only about 3 days, under optimal conditions. And hence allows quick experimental turn over and generation of large population.
4. **Transparent body-** this feature is particularly valuable for studying developmental processes and tracking fluorescently labeled molecules.
5. **Ease of culture and manipulation-** It is easy to cultivate and maintain in the laboratory,requireing minimal resources.

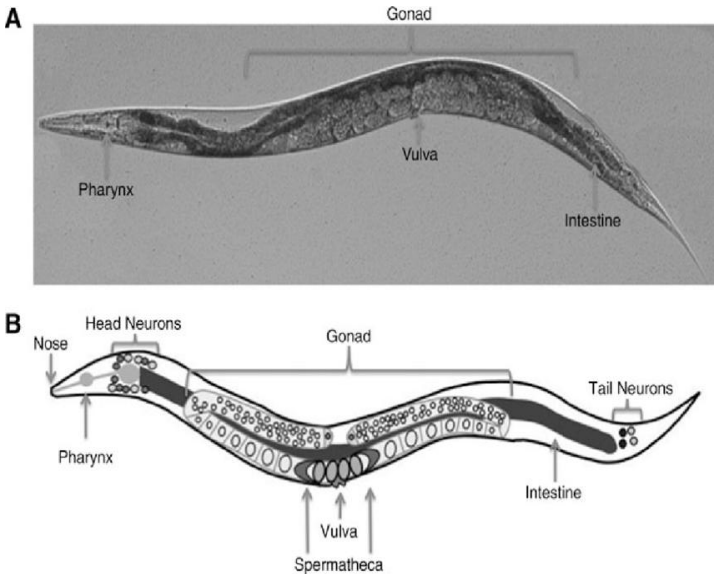


Fig.4 A picture and diagram of *Caenorhabditis elegans* labeled with important features. (A) Differential interference contrast image of an adult worm. (B) Schematic diagram of the major features found in adult worms.

It has been used to study various biological processes, including: Developmental biology, Neurobiology and behavior, Disease modeling (e.g.,

neurodegenerativediseases, cancer),Aging and longevity, Stress response and cellular physiology.

Its simplicity, genetic tractability, and conserved genetics make It is an attractive model organism for experiments to explore complex biological questions.

2.2. Life cycle

Embryogenesis of this soil-dwelling worm lasts for approximately 16 hr at 20°C and following fertilization a largely impermeable eggshell is produced, which allows the embryo to develop entirely free of the mother [11]. 8 The embryos are retained by the hermaphrodite until they are at the 24-cell stage and then are all laid together. The hermaphrodite embryo contains 558 nuclei at hatching and gives rise to an L1 larva. The worm eats and molts through four larval stages (L1-L4). The (L1) larvae stage lasts for 16 hours, its other stages last for 12 hours. Each stage terminates with a lethargus like molt to form a new cuticle. The old cuticle is molted at the end of the lethargus.

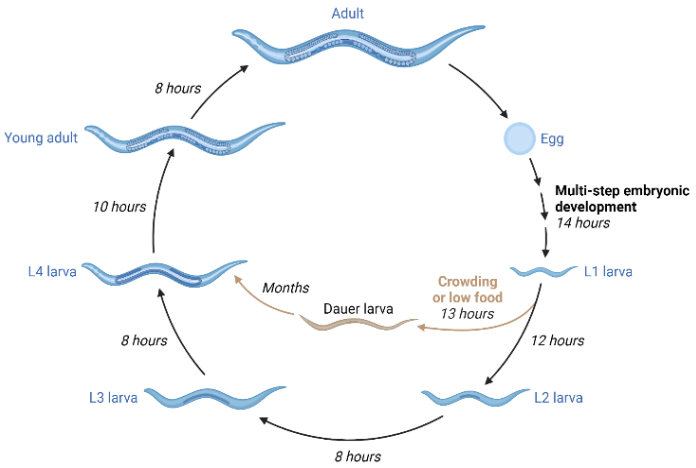


Fig.5: *C. elegans* Life cycle / BioRender Science Templates

After the final molt, adult hermaphrodites produce offspring for about 2 to 3 days when, presumably, they have none of their self-sperm left, but more offspring can be produced by sperm-depleted hermaphrodites that have mated with a male8. Hermaphrodite lifespan Hermaphrodites can live for quite a few weeks beyond their reproductive period before dying of senescence.

3. Review of Literature

COPPER is required for a variety of biological processes, such as mitochondrial respiration, antioxidant protection, and neurotransmitter metabolism. Disruption of copper homeostasis has been implicated in various neurodegenerative diseases, such as Alzheimer disease, Parkinson disease, and Menkes disease. (**Gaggelli, Kozlowski, Valensin, &Valensin, 2006; Kaler, 2011**). The copper-transporting ATPase ATP7A is also a major determinant of copper balance, and mutations in it result in menkees syndrome, a neurodegenerative disease resulting from deficiency of copper (**Horn & Tümer, 2000**; **Kaler, 2011**). ATP7A carries copper across the blood-brain barrier and in neurons, demonstrating the critical role of this protein in copper homeostasis in neurons (**Nyasae, Bustos, Braiterman, Eipper, & Hubbard, 2007**). In the same way, ATP7B, a second copper-transporting ATPase, is involved in Wilson's disease, in which copper accumulation

causes neurotoxicity (**Gaggelli et al., 2006**). The discovery underscores the delicate equilibrium that exists in copper homeostasis in the nervous system and its broader impact on neuronal function and neurodegeneration.

In the brain, copper is critical for activity of a number of enzymes, including cytochrome c oxidase, superoxide dismutase, and dopamine β-hydroxylase, which play roles in energy metabolism, antioxidant defense, and neurotransmitter biosynthesis (Lutsenko, Bhattacharjee, & Hubbard, 2010). Perturbation of copper homeostasis results in oxidative stress, mitochondrial and neurotransmitter dysregulation, all findings associated with neuronal diseases (Hare et al., 2012). As an example, in Alzheimer's disease copper serves to interact with amyloid-β peptides to promote aggregation and oxidative stress, and in Parkinson's disease c imbalance is the basis of Lewy body formation and dopaminergic neuronal damage (**Gaggelli et al., 2006; Hare et al., 2012)**. Such examples demonstrate the essential role of copper homeostasis in maintaining protein folding and preventing neuronal damage, which underlies widespread pathogenesis in neurodegenerative disease. Serotonergic neurons that synthesize and secrete the neurotransmitter serotonin (5-hydroxytryptamine, 5-HT) are highly sensitive to alteration in copper homeostasis. Serotonin is an important mood modulator, appetite regulator, and cognition regulator, whose synthesis is dependent on the tryptophan hydroxylase enzyme, which is an iron cofactor-requiring enzyme. Despite that, copper plays an indirect effect on serotonin signaling by regulating monoamine oxidase (MAO) activity, which is the enzyme involved in degrading serotonin and other monoamines (**Gaggelli et al., 2006**). The disruption of MAO function caused by deficiency or excess of copper will distort the level of serotonin and reduce proper neuronal transmission. This interconnection between the regulation of copper homeostasis and serotonin signaling puts emphasis on gaining knowledge regarding copper dysregulation effects on serotonergic neurons and how their function gets altered.

The nematode Caenorhabditis elegans (*C. elegans)* has turned out to be a convenient model organism to study the role of copper in neuronal function and neurodegeneration. Its well-characterized nervous system, genetic tractability, and reduced lifespan make it an ideal model system to study the effect of copper on serotonergic neurons and serotonin signaling **(Dubreuil, Marois, & Géminard, 2010; Fogle, Mobley, & Anbarchian, 2018**). In *C. elegans*, the feeding, locomotion, and egg-laying behaviors are regulated by the serotonergic neurons and, therefore, provide a good model to understand the influence of copper on serotonin-regulated behaviors. Experiments have demonstrated that chelation in this nematode with Cu causes dopaminergic neurodegeneration, and thus implies the same susceptibility for the serotonergic neurons **(Guo, Wang, Li, & Chen, 2020).** Further, these models have been significant in showing the molecular process under copper-induced neurodegeneration and neurotoxicity and also in revealing the conserved pathways involved with copper homeostasis **(Dubreuil et al., 2010; Fogle et al., 2018**).

It uses its copper homeostasis to be regulated through conserved molecular pathways that include the copper-transporting ATPases and metallothioneins, whose homologues can also be detected in human individuals (**Nyasae et al., 2007; Fogle et al., 2018**). These routes guarantee the correct delivery and storage of copper in cells to avoid its reaching toxic levels. For instance, these studies have proven that copper chelation rescues dopaminergic neurons from loss, demonstrating the therapeutic potential of copper modulation in neurodegenerative disease (**Guo et al., 2020).** Furthermore, this model have been employed to investigate copper-induced effects on protein misfolding and aggregation, which are key in the pathogenesis of neurodegenerative diseases like Alzheimer's and Parkinson's diseases (**Matlack, Tardiff, Narayan, & Lindquist, 2014**). These results show the effectiveness of this nematode as a model system to explore the function of copper in neurodegeneration and neuronal function.

It’s activity within serotonergic neurons and serotonin signaling is directly relevant to neurodegenerative disease. Dysregulation of copper has been suggested to cause a variety of psychiatric and neurological disorders, including depression, anxiety, and Parkinson's disease. Such diseases could be caused by copper dysregulation through modulating the level of serotonin and inhibiting serotonergic neurotransmission. For instance, oxidative stress caused by copper can be toxic to serotonergic neurons, decreasing serotonin production and transmission (**Gaggelli et al., 2006**). Cu may also interact with proteins associated with serotonin biosynthesis and breakdown, thus impairing serotonergic activity. It is therefore important to learn how copper affects serotonergic neurons in the development of treatment strategies for disorders related to serotonergic function.

Aside from the function in serotonergic neurons, copper homeostasis also plays a vital role in maintaining the function of other neurotransmitter systems, such as the dopaminergic and glutamatergic systems. For instance, copper is a cofactor for dopamine β-hydroxylase, an enzyme used in the synthesis of norepinephrine from dopamine (**Lutsenko et al., 2010).** Dysregulation of the level of copper thus has dramatic effects on neurotransmitter signaling that can contribute to the multifactorial pathophysiology of neurodegenerative diseases. The cross-talk with neurotransmitter circuits underscores the importance of having a complete understanding of the mechanisms of how copper controls neuronal function and neurodegeneration.

The function of copper in neuronal function and neurodegenerative disease has been well studied, and its relevance transcends serotonergic neurons to other neurodegenerative processes and cellular homeostasis. Dysregulation of copper impacts not only neurotransmitter systems but also protein misfolding, oxidative stress, and mitochondrial dysfunction, all of which are key components of the pathophysiology of

neurodegenerative disorders. As an example, copper was found to interact with amyloid-β peptides of Alzheimer's disease and encourage their aggregation and be involved in the generation of toxic oligomers (**Gaggelli et al., 2006**). And in Parkinson's disease, copper dysregulation has the effect of increasing the formation of Lewy bodies and dopaminergic neuronal destruction, once again exhibiting the metal's involvement in protein misfolding and neuronal damage (**Hare et al., 2012**). These results emphasize the significance of copper homeostasis to avoid the aggregation of misfolded proteins and resultant neurodegeneration in these disorders.

Apart from protein misfolding, copper is also a critical controller of oxidative stress in neurons. Copper may be involved in Fenton-like reactions, which lead to the formation of reactive oxygen species (ROS) that degrade cellular materials, such as lipids, proteins, and DNA (**Lutsenko et al., 2010**). This oxidative damage is especially difficult for neurons, which are active metabolically and therefore more vulnerable to ROS-induced harm. The dependency of the brain on copper-dependent enzymes, including superoxide dismutase, as an antioxidant defense makes this association even worse because both copper deficiency and overload can suppress the activity of the enzyme, generating heightened oxidative stress (**Hare et al., 2012**). This double function of copper-as inducer of ROS and as a constituent of antioxidant defense mechanisms-emphasizes the intricacy of cu homeostasis in neuronal disorder and health.

Use of this as a model has yielded valuable insights into molecular mechanisms governing copper-induced neurotoxicity and neurodegeneration. It have shown that chelation of copper is effective in suppressing dopaminergic progressive loss of nerve cells and thus present a promising therapeutic strategy for copper-induced neurodegenerative disease (**Guo et al., 2020).** Additionally, model have played a significant role in identifying conserved c homeostatic pathways such as the copper-transporting ATPases and metallothioneins, which also occur in human beings (**Nyasae et al., 2007; Fogle et al., 2018**). These pathways make sure of adequate distribution and storage of copper within cells to avert its harmful buildup. Tractability to genetics in *C. elegans* makes manipulation of these pathways easy, thus serving as an impressive tool to explore the impact of copper dysregulation on nervous system function and behavior.

Apart from this, to investigate copper's contribution to protein misfolding and aggregation, a cornerstone of the etiology of neurodegenerative disorders like Alzheimer's and Parkinson's (**Matlack et al., 2014**). *C. elegans*, for instance, has been used to demonstrate that copper accelerates amyloid-β peptide and α-synuclein aggregation and elicits neuronal malfunction and degeneration. These reports highlight the potential of *C. elegans* as a screening tool for compounds that modulate cu levels and inhibit protein misfolding, providing new therapeutic options for the treatment of neurodegenerative disease.

The cross-talk between copper homeostasis and neurotransmitter systems and not merely the serotonergic neurons but dopaminergic and glutamatergic systems as well. Cu is a cofactor of the enzyme dopamine β-hydroxylase for norepinephrine biosynthesis from dopamine and dysregulation in this can disrupt catecholamine signaling (**Lutsenko et al., 2010**). C also regulates the functioning of glutamate receptors, which are crucial for excitatory neurotransmission and synaptic plasticity. C dysregulation can cause excitotoxicity, a process that is involved in the pathogenesis of various neurodegenerative disorders, among them Alzheimer's and Parkinson's disease (Hare et al., 2012). The results of this study illustrate the ubiquity of copper dysregulation on neuronal function and emphasize the importance of understanding it in its entirety to account for its role in neurodegeneration.

In brief, copper plays a multigene function to have in neuronal function and neurodegeneration as it impacts mechanisms like protein misfolding, oxidative stress, and neurotransmitter signaling. Use of *C. elegans* as a model organism has not only yielded data concerning the molecular basis of copper-induced neurotoxicity but has also recognized potential treatments for copper-related neurodegenerative disease. Current research involving *C. elegans* models will also further shed light on the function of copper in neuronal function and neurodegeneration and open up avenues for the discovery of new therapeutic options. Through the integration of what is discovered with what is understood using mammalian models, scientists can gain a better understanding of the multifaceted relationship between copper homeostasis and neurodegeneration and eventually lead to the establishment of targeted therapy for copper-related neurological disorders.

4. Rationale

4.1. Copper deficiency has a powerful impact on the nervous system and interferes with essential processes of neurotransmitter production, antioxidant defenses, and mitochondrial function. All these interference points can lead to oxidative stress, defective synaptic transmission, and neurodegeneration and have been recorded in vertebrate and invertebrate models **(Locatelli & Farina, 2024; Gaier, Eipper, & Mains, 2013).**Previous studies in the laboratory demosntrates that intracellular copper regulates neurite outgrowth and neuronal viability of the PC-12 derived neurons (ref). Unpublished data from the laboratory demostrates that copper depletion causes degenration of the dopaminergic neurons and signalling in *C. elegans*. Inorder to further dissect the role of intracellular copper on other neurons, we investigated the effect of copper on the serotonergic neurons in *C. elegans*.

In vertebrates such as rodents, zebrafish, and humans, the nervous system is highly complex with complex networks of neurons and glial cells controlling behavior, cognition, and motor functions. Research in zebrafish has given important insights into CNS patterning and development and has pointed out the conserved function

of copper in neuronal development and function across all species (**Wilson, Brand, & Eisen, 2002**). In the same way, mammalian research has shown the synaptic effects of copper and its activity in regulating neurotransmitter release and synaptic plasticity (**Gaier, Eipper, & Mains, 2013).** Vertebrate nervous systems, however, generally remain too complex to allow for separation of individual molecular processes responsible for the action of copper.

As an alternative, *C. elegans* is an inexpensive simplistic model system that can be used to examine metal biology and neuron function. *C. elegans* provides a system that can be utilized to study the effects of copper deficiency on specific neuronal populations and behaviors since it has a limited number of well-characterized neurons i.e. 302 (**Paridaen & Huttner, 2013**). *C. elegans* is an extremely suitable model organism to study the mechanisms of copper function in neuronal health because it has rudimentary neuronal circuits and metal-regulatory mechanisms that are common to vertebrates.

4.2. Taking advantage of the genetic tractability and simplicity of *C. elegans*, this study seeks to elucidate the effects of copper deficiency on defined subpopulations of neurons and their related behaviors. In so doing, the findings not only will contribute to our knowledge of the role of copper in neurons but also will inform the wider significance of copper dysregulation for neurodegenerative disease.

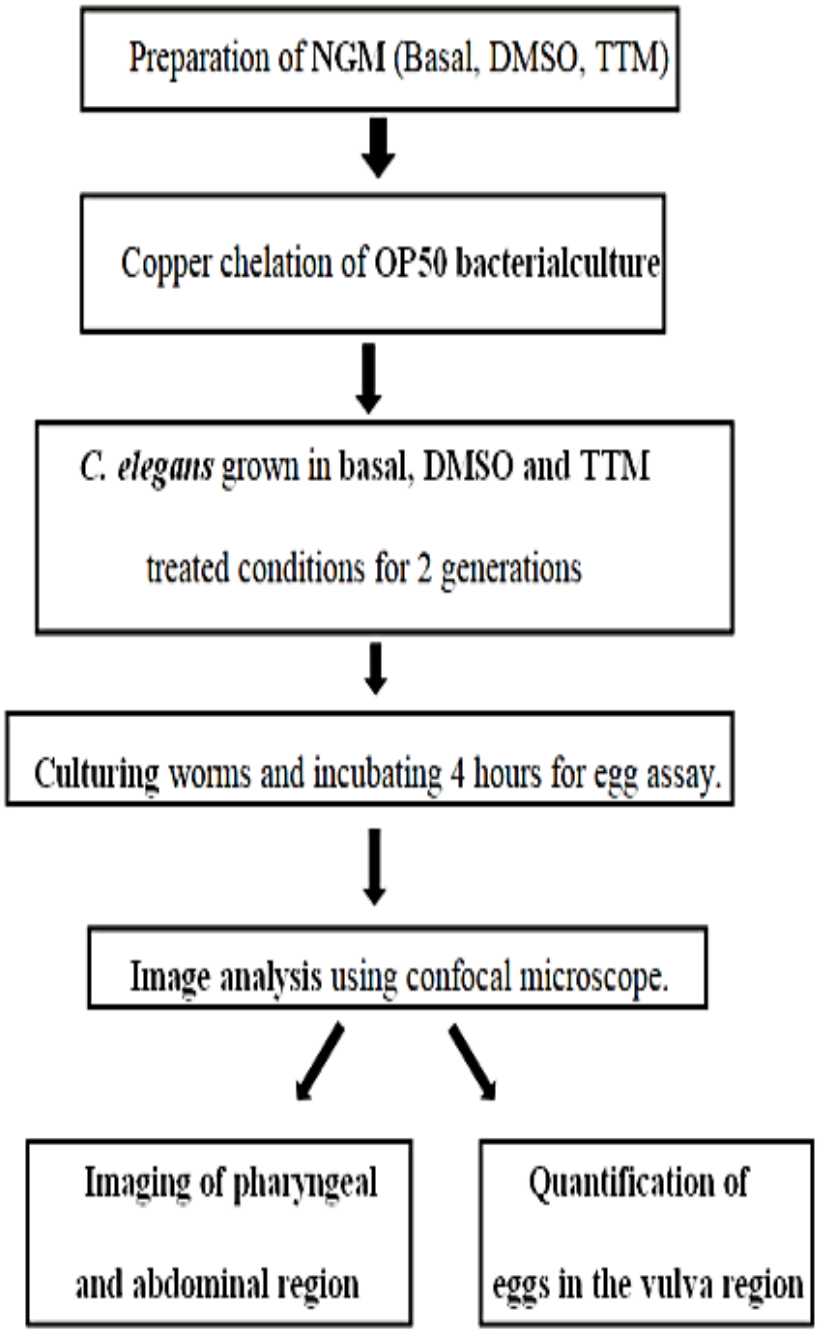
5. Objective of the study

5.1.The prime objective is to evaluate the effect of copper chelation on pharyngealADF,NSM) and abdominal (HSN) serotonergic neurons in the *C.elegans* ***mgIs-42 strain*****.**

5.2.To examine the effect on specific serotonergic neurons on the corresponding behaviour.

6. Materials and Methods

6.1. Experimental strategy:


Preparation of NGM (Basal, DMSO, TTM)
Copper chelation of OP50 bacterialculture
C. elegans grown in basal, DMSO and TTM treated conditions for 2 generations
Culturing worms and incubating 4 hours for egg assay.
Image analysis using confocal microscope.
Imaging of pharyngeal and abdominal region
Quantification of eggs in the vulva region

Maintenance of *C. elegans*

Worms are typically raised on agar plates seeded with a lawn of the bacterium Escherichia coli in the laboratory. When the bacteria are gone, the worms are living off their fat. Deprived of food, the growth of young larva worms is halted. When stocks do not need regular feeding, the worms can endure (worms have been known to survive at least one month [often starved plates are still useful at 15° for up to 6 months]; thus, when a stock doesn""t need to be continually fed), cruciably. 8 If you need healthy, worms. A little of the agar of the old plate can be transferred to a new plate, where there are bacteria. The worms crawl to fresh bacteria and grow on.

Some additional traits are also critical to maintaining C. elegans stocks and using them in an experiment. First, due to its self-fertilizing hermaphroditism, one worm can reproduce and fill a plate. Second, worm colonies can be frozen and kept in suspended animation for years and then revived. Third, worms are small and many can be grown in a small space. 4th, worms can be reared at mycierit murm

6.2.**Preparation of growth media (NGM) for *C. elegans***:

Ingredients for 150ml Nematode Growth Media (NGM):-

Table 1

Component	Quantity
Agar	2.55 g
NaCl	0.45 g
Distilled Water	146.25 ml

Autoclave the media, cool to 55°C in a water bath and add sterilized supplements .

Additive	Quantity
1 M $CaCl_2$	150 µl
1 M $MgSO_4$	150 µl
KPO_4 Buffer	3.75 ml
5 mg/ml Cholesterol (EtOH)	150

6.3.**Copper chelation**

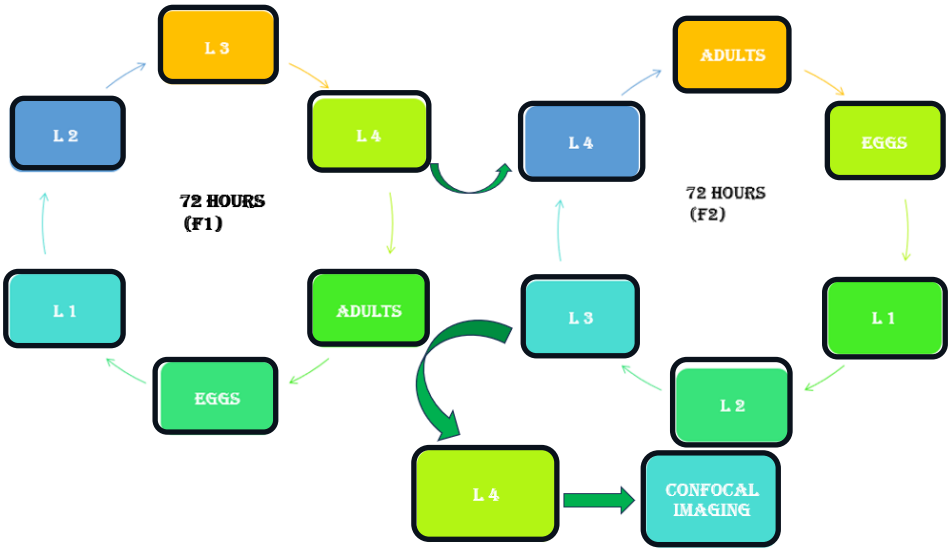


Fig.6: *mgIs-42 strain*of *C. elegans* is grown for 2 generations in Basal, DMSO and Copper chelated TTM treated conditions before confocal imaging.

The worms were first staged at L4 stage and allowed to grow for one generation in 250µM TTM and fed with TTM treated OP50 bacteria. After 72 hours, the new generation L4 (F1) worms were picked up and plated in fresh TTM plates and grown for another generation till they reached the L4 stage (F2). These (F2) generation L4 worms which ultimately turns into adult are used for imaging under confocal microscope.

6.4.**Confocal microscopy and image analysis**

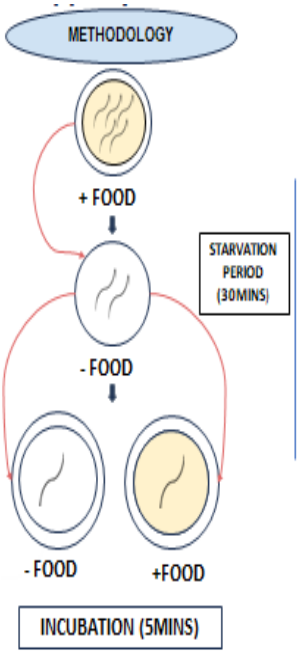
In confocal microscope we see the pharyngeal and abdominal regions to analyse the impact of Cu^{+} chelation on serotonergic neurons(ADF,NSM and HSN). Worms are imaged using Olympus evident spinning ,60x oil immersion lens.Before imaging worms are first washed with M9 buffer. Then sodium azide is put to immobilize the worms. This creates temporary paralysis in worms and allows smooth imaging.The purpose is to investigate how serotonergic neurons basically looks like in basal condition under confocal microscope and then analyse how TTM responsible for causing copper deprivation affects these serotonergic neurons in terms of their structural integrity.In this nematode, the serotonergic neurons control feeding, locomotion, egg-laying, stress response, and learning.

6.5.**Investigating the Response**

For examining the enhanced slowing response in *C. elegans*, their populations were synchronized and cultured on nematode growth medium (NGM) which is, supplemented with *E. coli OP50* as food source. The worms were divided into three treatment groups:

Basal condition: Worms grown on NGM with no additional treatment.

DMSO condition: To account for solvent control in copper chelation investigations, worms are cultivated on NGM plates treated with DMSO.



Method followed in our lab for investigating enhanced slowing response.

TTM condition: Copper-deficient conditions were obtained by culturing in **250 µM tetrathiomolybdate (TTM)**, a copper chelator), and supplied with *OP50* grown in the influence of TTM.

Each group was further subdivided and tested under **two environmental contexts**. Worms are transferred to plates with (+) or without (-) *OP50*.The enhanced slowing response was measured in terms of body bends per 60 seconds and observed by placing worms on assay plates and tracking their movement under a stereomicroscope. Body bends were manually counted. The enhanced slowing response, which is mostly

mediated by pharyngeal serotonergic neurons, was defined as a marked decrease in body bending when food is present as opposed to absence of food.

At least 20 worms were analyzed under each condition, and data were compared statistically to ascertain significant differences, and results were expressed as mean ± SEM. These techniques offer a comprehensive assessment of the improved slowing response under different environmental and chemical conditions.

Table 2.

Condition	Food Status	Enhanced Slowing Observed	Interpretation
Basal	+ / -	☐	Normal response; serotonin signaling intact
DMSO (Control)	+/ -	☐	No interference from solvent; normal neuronal function
TTM (Copper chelation)	+ / -	☐	Copper deprivation does not impair pharyngeal neurons

6.6.**Investigating the egg laying behavior**

When we check different serotonergic neurons like ADF and NSM in the pharyngeal region and HSN in the abdominal region then we move towards analysing the corresponding behaviours for which these neurons are responsible.In C. elegans, the serotonergic neurons control feeding, locomotion, egg-laying, stress response, and learning.

Table 3.

Type of Neuron	Location	Function
ADF (***Sensory neuron***)	**Anterior(head,near pharynx)**	**Chemosensation**
NSM (***Neurosecretory Motor Neurons)***	**Anterior (pharynx)**	**Feeding, locomotion and stress response(starvation)**

HSN (***Hermaphrodite-Specific Neurons)***	**Posterior (tail, near vulva)**	**Stimulates egg-laying by activating vulval muscles.**

This lab has initially done a research on the Enhanced Slowing Response to check the effect of copper deprivation on locomotion but they have not found any significant change which forms a hypothesis that pharyngeal neurons are not much affected. So now our lab is mainly focusing on investigating the egg laying behaviour. As we know HSN neurons are responsible for stimulating the egg laying behaviour. It secretes serotonin which in turn activates the vulval muscles by binding to serotonin receptor (SER 1). My research mainly focused on the abdominal serotonergic neurons i.e. the HSN neurons .

7. Results and Discussions

7.1. Results

a. Confocal microscopy of tph-1:: GFP (*mgIs-42*)

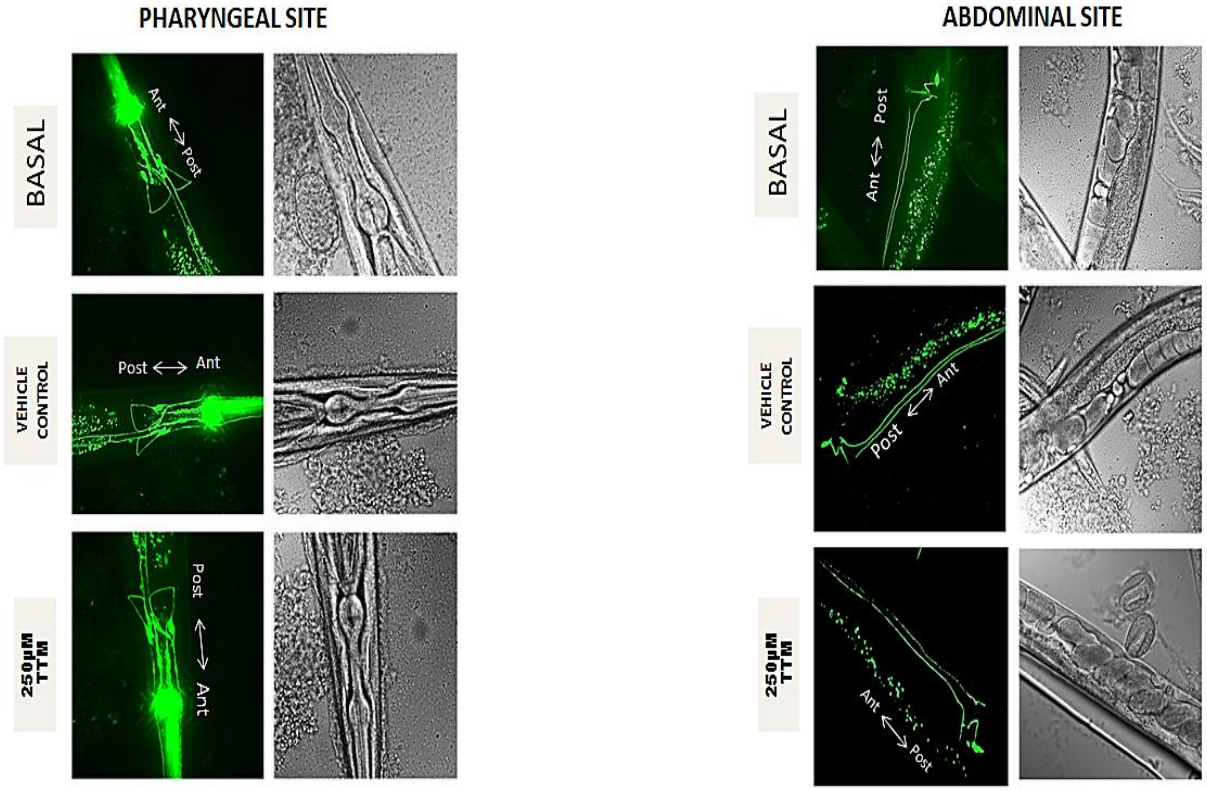


Fig.7: **Panel a**: represents the anterior and posterior pharyngeal portion of Basal,DMSO and TTM treated Tph1:GFP*C. elegans* (***mgIs-42 strain***), respectively under 60x oil immersion.

Fig 8: Panel b: represents the anterior and posterior abdominal portion of Basal,DMSO and TTM treated Tph1:GFP*C. elegans* (***mgIs-42 strain***), respectively under 60x oil immersion.

TTM treated adult worms were imaged using **Olympus evident spinning** confocal microscope **,60x oil immersion lens** to investigate the visible effects of copper deprivation. Imaging results demonstrate no effect on the pharyngeal NSM and ADF neurons (Fig 7). This demonstrates that copper deprivation does not have any visible effect on these neurons. However, copper deprivation shows degenerative features in the abdominal HSN neurons (Fig 8). The axonal extension of the HSN neuron appears broken in the presence of TTM.

b. Enhanced slowing Response

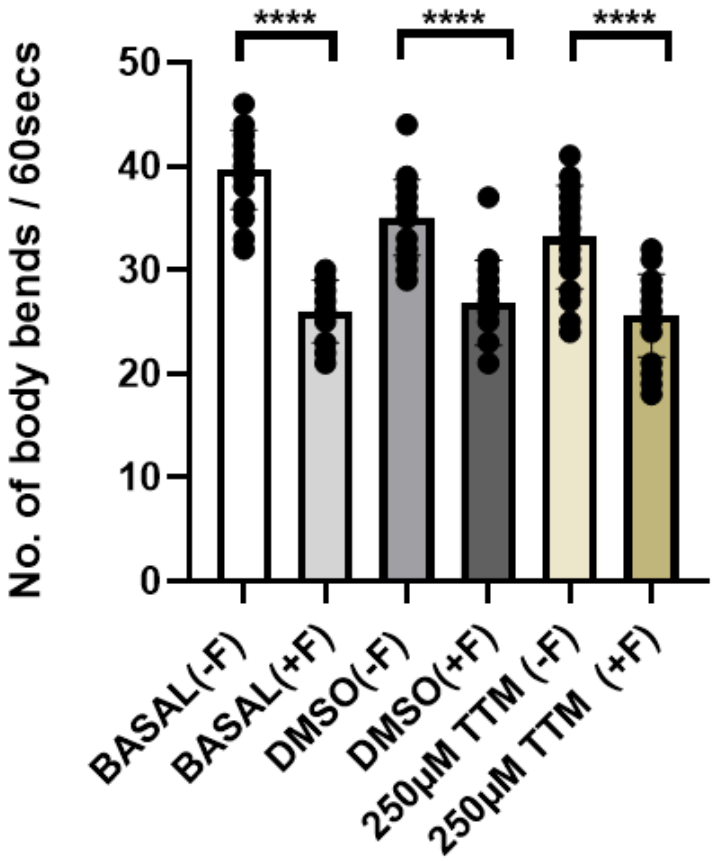


Fig- 9. Graph showing statistical representation of Enhanced Slowing Response using N2 bristol strain.

Next we investigated potential of the worms to execute enhanced slowing which is largely regulated by the pharyngeal serotonergic neurons. Enhanced slowing is investigated as described in the methods section. Data is represented as body bends over 60 seconds under various conditions. The control worms show 40 mean body bends per 60 seconds in the absence of food. This movement decreases in the presence of food to mean body bends of 26 per 60 seconds. Similarly, the worms show mean body bends of 26 per 60 seconds in presence of food and 36 in absence of food when grown in the presence of DMSO. There is statistically significant reduction of body bends in the presence of food under both the condition (fig 9). The worms show 25 body bends per 60 seconds in the presence of food and 35 per 60 seconds in the absence of food under TTM treatment. The worms can decrease their body bends in presence of food even under copper deprivation. In other words, copper deprivation does not affect the functional capacity of the worms to perform enhanced slowing. This conforms with the imaging results presented in the previous section.

Copper deprivation has no impact on pharyngeal neurons. As an outcome the worms do not lose their ability to perform the enhanced slowing activity which is largely regulated by the pharyngeal neurons.

c. Egg Assay

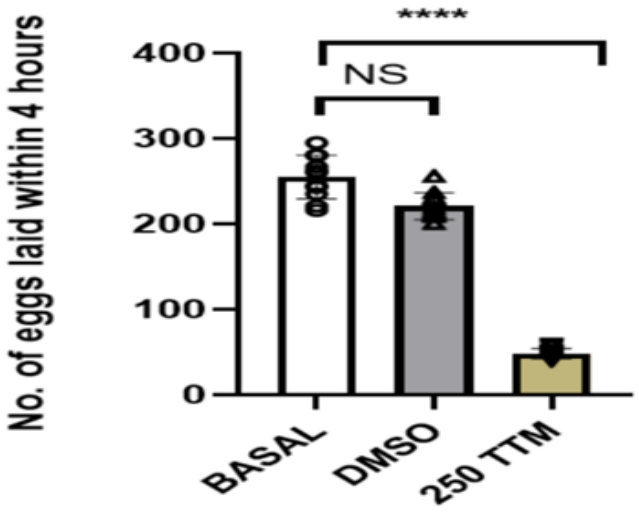


Fig 10. The graphs show statistically calculated data representing the quantification of egg under Basal, DMSO and TTM treated conditions.(Unpublished data)

After that, we investigated if the degeneration of the HSN neuron under copper deprivation affects the egg laying behaviour of the worms. Secretion of serotonin by the HSN neuron into the vulval muscles is essential for the contraction of the vulval muscles. The data represents a significant amount of decrease in the no. of eggs laid by the adult *C.elegans*, when observed after incubation period of 4 hours under TTM treated condition. The control and DMSO treated worms lay 240 and 220 eggs respectively in 4 hours (Fig 10). However worms grown under the influence of TTM lay 40 eggs in 4 hours (Fig 10). Therefore, TTM treatment has led to a significant reduction in egg laying by the worms. We know that HSN neurons are located in the abdominal region and it secretes serotonin which has a great impact in stimulating the vulval muscles. This stimulation further leads to its activation and modulation of vulval contractions (**Fogle, K. J., Mobley, J. L., & Anbarchian, T**.).The chelation leads to degeneration of serotonergic HSN neurons which affects corresponding egg laying .

7.2. Discussion

The result indicates, selective degeneration of the abdominal HSN neurons. Pharyngeal neurons are unaffected by copper deprivation via TTM **(Fig 8**).But the abdominal neurons specifically HSN neurons, are degenerated(**Fig 9**). This finding highlights the varying resistance of neuronal populations to copper deprivation, suggesting that some neurons may be more reliant on copper-dependent processes or may have special differences in how they handle the copper deficit condition.Due to special metabolic needs or compensatory mechanisms that make up for copper depletion, pharyngeal neurons may be inherently more resilient. Pharyngeal neurons, for instance, might express more copper-independent antioxidant enzymes to counteract copper deprivation.On the other hand, it was seen that the hermaphrodite i.e hsn neurons that are crucial for egg laying behaviour rely heavily on copper for both survival and function. C is needed for functioning of major enzymes subsisting on neuronal health like superoxide dismutase and dopamine-beta-hydroxylase.The degeneration of HSN neurons is most likely to hinder either the pathway of serotonin-induced signalling channels or the flow between multiple neuronal pathways as far as there are behavioral deficits.. This finding lays a strong foundation for an investigation into how copper affects neuronal survival and function, taking specific vulnerabilities of a particular area into account.

Copper's function in reproductive physiology is further supported by the observed sharp decline in egg-laying behavior. Serotonin, a major modulator of vulval muscle contractions, is produced and released by the HSN neurons.Deprivation of cu can cause neuronal death in HSN and finally impair serotonin signaling that would cause infrequent and ineffective egg-laying. In addition to this c is a cofactor of energy metabolism and, accordingly, it would exacerbate this decline of egg production by limiting the amount of energy that organisms might have for reproductive processes. Decreased number of eggs under copper deficiency conditions would, therefore, affect reproductive fitness in *C. elegans*. Thus, it implies that the levels of copper in ecology and diet leave a mark on the population dynamics and, hence, evolutionary strength of the organism in real life. This study should proceed to discover whether copper supplementation can restore HSN neurons' functions and reverse the egg-laying defect, thus providing some hints about a possible therapeutic avenue into reproductive disorders related to copper intoxication.

8. **Conclusion**

This study emphasizes the differential susceptibility of Cu deficiency in *C. elegans* neuronal populations, where there is a clear dichotomy in their responses. Pharyngeal neurons, i.e., ADF and NSM, are highly resilient towards copper chelation, and their structure integrity and function remain unchanged without any discernible alteration in morphology and behavior. This is also evident from the unaffected enhanced slowing response, a pharyngeal neuron-mediated behavior, showing that these neurons have strong compensatory mechanisms to overcome copper deficiency.*C. elegans* possesses a clear behavioral response to the presence & absence of food, with less body bends when food is absent and more movement when food is present. The enhanced slow responsiveness is not altered by cu deficiency, indicating that the underlying processes are strong and copper-independent. This is a sign of the nematode's adaptability to the environment.

Conversely, abdominal neurons, especially the serotonergic HSN neurons, show dramatic degeneration in copper deficiency. The degeneration is correlated with a drastic decrease in egg-laying behavior, highlighting the critical function in the survival & integrity of HSN neurons. As regulators of serotonin synthesis, HSN neurons play crucial role in regulation of vulval muscle contractions necessary for egg-laying. Degeneration of these neurons in copper deficiency disables this process, resulting in abnormal reproductive behavior.These observations not only demonstrate the significance of copper in supporting neuronal and reproductive function but also provide a basis for further exploration of the molecular mechanisms of neuronal susceptibility to copper deficiency.

Subsequent studies may determine if copper supplementation can rescue HSN neuronal function and egg-laying behavior, with therapeutic significance for copper-related reproductive disease. Identification of compensatory mechanisms in pharyngeal neurons also may uncover novel targets for the improvement of neuronal resistance to environmental toxins. Overall, this research continues to shed light on the role of copper in neuronal health and behavior, with general relevance to trace metal biology and reproductive physiology.

This research has made the most significant link between metal ion homeostasis and activity of serotonin-producing neurons. in *C. elegans* that forms the basis for understanding trace metal impact on neurotransmitter systems. These results have significant relevance in neurodevelopmental disorders, environmental toxicology, and neurodegenerative disease, where dysregulation of copper and serotonin signaling is now being set up as fundamental component. The lab will build on this foundation to investigate mechanistic, behavioral, and translational components of copper-serotonin interaction.

Dysregulation of copper is common in neurodevelopmental and neuropsychiatric illnesses including Wilson's disease, Menkes disease, and autism spectrum disorder. This study outlines a mechanistic model towardshow copper regulates serotonin signalling and gives some potential targets for the disorders.

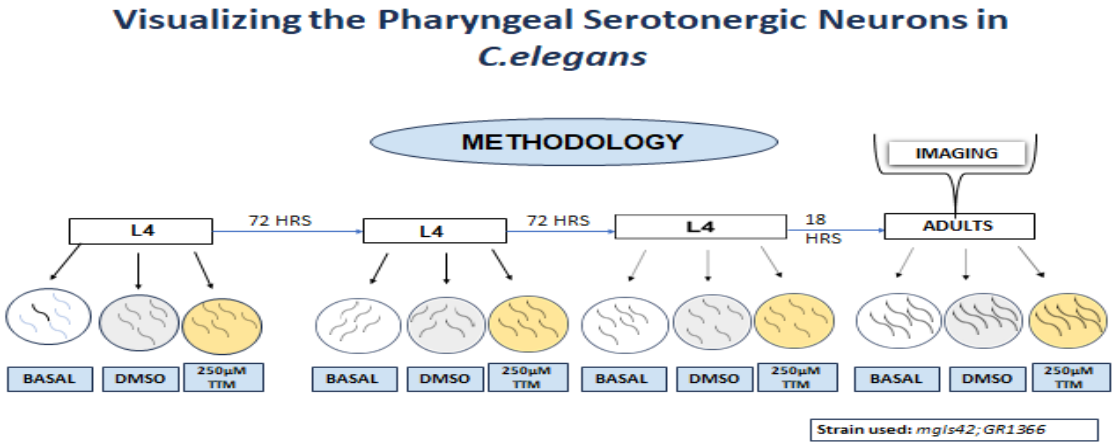
Since it can serve as an environmental toxin, its effects on neurotransmitter systems remain to be comprehensively understood. This study provides the foundation for the assessment of exposure-related neurotoxicity, applicable towards environmental and public health.

Their deposition is a hallmark of aging and neurodegenerative diseases like Alzheimer and Parkinson disease. By outlining the function of copper in serotonin signalling, the present study may reveal new mechanisms of these diseases and provide direction toward intervention.

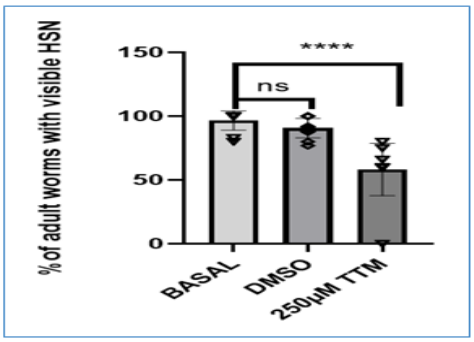
9. **Future Research Directions**

The lab will undertake a series of specific experiments to further explore copper's biological significance in serotonin signalling and the ramifications of these interactions more broadly. The selective effect on HSN neurons emphasize the differential effect on different neurons which needs to be explored further. Future lab experiments involve production of a cross of chca-1::mTFP and tph-1::RFP lines. The strategy is to visualize trace metal-responsive cells in relation to serotonergic neurons, especially HSN, with fluorescent markers.Including this protocol included in future experiments represents a step toward combining genetic labeling with live imaging to provide better insight into trace elements' ability to control neurotransmitter function. This combined strategy will enable the lab to monitor dynamic changes in neuronal environments and evaluate their influence on behavior with spatial and temporal resolution. Together, these studies will advance the mechanistic underpinnings of elemental regulation in neural circuits and could reveal novel intersections between metal homeostasis and neuromodulatory processes.

10. **Appendix**



Cu(I) depletion significantly reduced the number of adult worms with intact HSN



Confocal microscopy at IISER,Kolkata

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